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METHOD FOR DOWN-REGULATING OSTEOPROTEGERIN LIGAND ACTIVITY

FIELD OF THE INVENTION

The present invention relates to improvements in therapy and
prevention of osteoporosis and other diseases characterized by
5 continued loss of bone tissue. More specifically, the present
invention provides a method for down-regulating osteoprotegerin
ligand (OPGL) by enabling the production of antibodies against
OPGL in subjects suffering from or in danger of suffering from
osteoporosis. The invention also provides for methods of produ-
10 cing modified OPGL useful in this method as well as for the
modified OPGL as such. Also encompassed by the present invention
are nucleic acid fragments encoding modified OPGL as well as
vectors incorporating these nucleic acid fragments and host
cells and cell lines transformed therewith. The invention also
15 provides for a method for the identification of OPGL analogues
which are useful in the method of the invention as well as for
compositions comprising modified OPGL or comprising nucleic
acids encoding the OPGL analogues.

FIELD OF THE INVENTION

20 Osteoporosis is a major and growing health problem worldwide. It
affects an estimated 75 million people in the United States of
America, Europe and Japan combined. Thus, it is the most common
systemic bone disorder in the industrialised part of the world.

Osteoporosis affects one in four postmenopausal women and a
25 majority of the elderly, including a substantial number of men.
The cost of osteoporosis in the United States of America with 15
million affected people was estimated to be 3.8 billion USD
annually in 1984. This translates by extrapolation to a world-
wide cost of something in the order of at least 20 billion USD.

Osteoporosis is a systemic skeletal disease characterised by low
bone mass and micro-architectural deterioration of bone tissue,
with a consequent increase in bone fragility and susceptibility
to fractures. Although all bones are affected, fractures of the
5 spine, wrist and hip are typical and the most common. The risk
of developing osteoporosis increases with age and is higher in
women than in men. Its etiology appears to reside in the mechan-
isms underlying an accentuation of the normal loss of bone mass,
which follows the menopause in women and occurs in all individu-
10 als with advancing age.

Peak bone mass is achieved at about 35 years of age. After
reaching its peak, bone mass declines throughout life due to an
imbalance in remodelling. Bones lose both mineral and organic
matrix but retain their basic organisation.

15 Bone consists of a mineralised extracellular matrix composed of
a variety of proteins and proteoglycans; the principal component
being type I collagen. The mineral encrusting the extracellular
matrix is hydroxyapatite $(Ca_3(PO_4)_2 \cdot Ca(OH)_2)$. Bone is continuously
modelled during growth and development and remodelled throughout
20 life in response to physical and chemical signals.

The growth, development and maintenance of bone are highly
regulated processes, which at the cellular level involves the
co-ordinate regulation of bone-forming cells (osteoblasts) and
bone-resorbing cells (osteoclasts). The level of bone mass
25 reflects the balance of bone formation and resorption.

Osteoblasts arise from mesenchymal stem cells and produce bone
matrix during development, after bone injury, and during the
normal bone remodelling that occurs throughout life. Osteoclasts
differentiate from hematopoietic precursors of the monocyte-
30 macrophage lineage and resorb bone matrix.

An imbalance of osteoblast and osteoclast functions can result in the skeletal abnormalities characterised by increased bone mass (osteopetrosis) or by decreased bone mass (osteoporosis).

Studies of osteopetrosis in mutant mice have shown that genetic
5 defects in osteoclast development, maturation, and/or activation lead to decreased bone resorption and uniformly result in severe osteopetrosis (Marks, 1989). Nevertheless, relatively little has so far been known about the soluble factors that act physiologically to regulate osteoclast development.

10 Recently, however, two proteins that take part in this regulation have been described and characterized (Simonet et al., 1997; Lacey et al., 1998). These two proteins are osteoprotegerin and osteoprotegerin ligand.

Osteoprotegerin is a novel secreted member of the tumour necro-
15 sis factor receptor family. In vitro, osteoprotegerin blocks osteoclastogenesis in a dose dependent manner. Transgenic mice expressing osteoprotegerin exhibit a generalized increase in bone density (osteopetrosis) associated with a decrease in osteoclasts. Administration of recombinant osteoprotegerin
20 produces similar effects in normal mice and protects against ovariectomy-associated bone loss in rats (Simonet et al., 1997). In addition, osteoprotegerin-deficient mice (knock out mice) while normal at birth develop early onset osteoporosis and arterial calcification (Bucay et al., 1998). These observations
25 strongly point to the possibility that osteoprotegerin blocks the differentiation of osteoclasts, the principal if not sole bone-resorbing cell type, suggesting that it can act as a humoral regulator of bone resorption. Osteoprotegerin is the subject matter of WO 97/23614.

30 It was hypothesized that osteoprotegerin may exert its effect by binding to and neutralising a factor that stimulates osteoclast

development, thus inhibiting osteoclast maturation (Simonet *et al.*, 1997).

Osteoprotegerin ligand (OPGL) is a novel member of the tumour necrosis factor family of cytokines that exists in both a
5 membrane-bound and a soluble form. OPGL binds to osteoprotegerin with a binding affinity of 4 nM. *In vitro*, OPGL activates mature osteoclasts and modulates osteoclast formation from bone marrow precursors in the presence of CSF-1. It has also been demonstrated that OPGL binds to the surface of osteoclast progenitors
10 in CSF-1-treated bone marrow. The receptor for OPGL on these hematopoeitic progenitor cells is, however, unknown. Recombinant soluble OPGL is a potent inducer of bone resorption *in vivo* (Lacey *et al.*, 1998).

Description of OPGL

15 OPGL is synthesised as a type II transmembrane protein consisting of 317 amino acid residues (human, cf. SEQ ID NO: 2) or 316 amino acid residues (murine, cf. SEQ ID NOs: 4 and 6). Alignment of the two amino acid sequences show that identical amino acid residues are found at 87% of the homologous positions.

20 The OPGL amino acid sequence contains a short cytoplasmic domain in the N-terminus followed by the putative transmembrane region between amino acid residues 49 and 69. Based on its homology to tumour necrosis factor alpha, the extracellular part of OPGL has been suggested to be comprised by two domains: a stalk region
25 extending from amino acid residue 70 to 157, and the active ligand moiety extending from amino acid residue 158 to the C-terminus.

The most closely related protein to OPGL appears to be the apoptosis inducing cytokine TRAIL with less that 25% identical
30 amino acid residues. OPGL has also very recently been cloned in

other contexts and was called TRANCE (Wong *et al.*, 1997, J. Biol. Chem. **272**: 25190-25194) and RANKL, respectively (Anderson *et al.*, 1997, Nature **390**: 175-179.

Several N-terminal deletion variants of murine OPGL have been
5 expressed in *E. coli* and purified. These variants consisted of amino acid residues 75-316, 128-316, 137-316, and 158-316, respectively. The three shortest variants had similar β-sheet structure based on circular dichroism studies, and all were able to bind to osteoprotegerin. More important, though, is that the
10 three variants were active in *in vitro* assays (Lacey et *al.*, 1998).

The shortest variant was studied further. Like tumour necrosis factor alpha, this variant OPGL exists as a trimer in solution and forms 3:3 complexes when incubated with osteoprotegerin. The
15 binding affinity was found to be 4 nM. This variant induces significant increases in blood ionized calcium (hypercalcemia) in mice *in vivo*. Co-administration of osteoprotegerin significantly reduced this hypercalcemic effect of OPGL.

The longest variant (amino acid residues 75-316) of OPGL did not
20 bind to osteoprotegerin and it did not have any biological activity.

At the time of construction of the N-terminal deletion variants the natural cleavage site in OPGL was not known. Expression of full-length OPGL in human 293 fibroblasts resulted in soluble
25 OPGL beginning at amino residue 139 in the murine protein or at the homologous amino acid residue 140 in the human protein. These expression studies also showed that soluble OPGL resulting from expression in human cells is glycosylated. This is not surprising as both murine and human OPGL contain three potential
30 N-glycosylation sites in the C-terminal ligand domain.

The concentrations of osteoprotegerin in blood and tissues are not known but the protein has significant biological activity at a concentration of 1 ng/ml.

Biological activity of OPGL

5 OPGL is a potent osteoclast differentiation factor when combined with CSF-1. Neither of these components alone are capable of inducing osteoclast differentiation from progenitor cells.

OPGL is a potent activator of mature osteoclast. On its own, OPGL activates mature osteoclasts to resorb bone. OPGL has not

10 been observed to act as an osteoclast growth factor or osteoclast survival factor in these experiments.

The action of OPGL does not seem to be species restricted as murine OPGL also induced osteoclast formation in cultures of human peripheral blood mononuclear cells.

15 SUMMARY OF THE INVENTION

We find that the above-referenced data suggests a pathophysiological role of OPGL. The *in vivo* evidence is partially circumstantial or indirect but is in our opinion convincing especially in combination with the direct evidence.

20 Observing that injection into mice of the recombinant C-terminal domain of OPGL results in severe hypercalcemia in our opinion points directly to a pathophysiological role.

Indirect evidence comes from the osteoprotegerin-deficient mice (knock out mice) that even though normal at birth develop early

25 onset osteoporosis. This shows that removing a protein that binds OPGL and neutralises its effects leads to osteoporosis. We

conclude that the most likely reason for this is an increased osteoclast maturation and activation caused by OPGL.

Two other pieces of indirect evidence are that both mice trans-genic for osteoprotegerin and mice injected with recombinant
5 osteoprotegerin develop osteopetrosis. This shows that unnatural high levels of a protein that binds OPGL and neutralises its effects leads to osteopetrosis. Here, we conclude that this has its reasons in a decreased osteoclast maturation and activation caused by neutralisation of OPGL.

10 We therefore suggest a model in which OPGL and osteoprotegerin act as positive and negative regulators of osteoclast develop-ment, respectively. In other words OPGL promotes bone resorption while osteoprotegerin inhibits bone resorption.

Thus, in relation to osteoporosis OPGL could be thought of as
15 "the bad guy" promoting the bone resorption that in the end leads to osteoporosis. Likewise osteoprotegerin can be visual-ised as "the good guy" trying to fight "the bad guy" through neutralisation of its effects.

We hence propose to down-regulate osteoclast differentiation/ma-
20 turation/formation and osteoclast activation through *in vivo* production of antibodies capable of neutralizing OPGL, thereby providing a safe and efficient means for treating/ameliorating and/or preventing osteoporosis and other diseases characterized by an excess rate of bone resorption compared to the rate of
25 bone formation.

Thus, in its broadest and most general scope, the present inven-tion relates to a method for *in vivo* down-regulation of osteoprotegerin ligand (OPGL) activity in an animal, including a human being, the method comprising effecting presentation to the
30 animal's immune system of an immunologically effective amount of

- at least one OPGL polypeptide or subsequence thereof which has been formulated so that immunization of the animal with the OPGL polypeptide or subsequence thereof induces production of antibodies against the OPGL polypeptide, and/or

5 - at least one OPGL analogue wherein is introduced a modification in the OPGL polypeptide which has as a result that immunization of the animal with the analogue induces production of antibodies against the OPGL polypeptide.

The most attractive aspect of this approach is that e.g. osteo-
10 porosis can be controlled by periodic but not very frequent immunizations, in contrast to a therapeutic approach which involves administration of osteoprotegerin or molecules having a binding affinity to OPGL analogous therewith. It is expected that 1-4 annual injections with an immunogenic composition will
15 be sufficient to obtain the desired effect, whereas administration of osteoprotegerin or other inhibitors of OPGL activity would require daily administrations.

The invention also relates to OPGL analogues as well as to nucleic acid fragments encoding a subset of these. Also
20 immunogenic compositions comprising the analogues or the nucleic acid fragments are part of the invention.

The invention also relates to a method of identifying analogues of OPGL as well as a method for preparing composition comprising the OPGL analogues.

25 Finally, the invention relates to a method treating osteoporosis and other diseases characterized in excess bone resorption, wherein is administered a non-OPGL molecule (typically an antibody) which blocks the interaction between OPGL and its receptor on osteoclast cells.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present specifi-
cation and claims will be defined and explained in detail in
5   order to clarify the metes and bounds of the invention.

The terms "T-lymphocyte" and "T-cell" will be used interchange-
ably for lymphocytes of thymic origin which are responsible for
various cell mediated immune responses as well as for helper
activity in the humoral immune response. Likewise, the terms "B-
10 lymphocyte" and "B-cell" will be used interchangeably for
antibody-producing lymphocytes.

An "OPGL polypeptide" is herein intended to denote polypeptides
having the amino acid sequence of the above-discussed OPGL
proteins derived from humans and mice (or truncates thereof
15 sharing a substantial amount of B-cell epitopes with intact
OPGL), but also polypeptides having the amino acid sequence
identical to analogues of these two proteins isolated from other
species are embraced by the term. Also unglycosylated forms of
OPGL which are prepared in prokaryotic system are included
20 within the boundaries of the term as are forms having varying
glycosylation patterns due to the use of e.g. yeasts or other
non-mammalian eukaryotic expression systems. It should, however,
be noted that when using the term "an OPGL polypeptide" it is
intended that the polypeptide in question is normally non-
25 immunogenic when presented to the animal to be treated. In other
words, the OPGL polypeptide is a self-protein or is an analogue
of such a self-protein which will not normally give rise to an
immune response against OPGL of the animal in question.

It should be noted that the use as a vaccine in a human of e.g.
a canine analogue of human OPGL can be imagined to produce the
desired immunity against OPGL. Such use of an xeno-analogue for
immunization is also considered part of the invention.

5 The term "polypeptide" is in the present context intended to
mean both short peptides of from 2 to 10 amino acid residues,
oligopeptides of from 11 to 100 amino acid residues, and
polypeptides of more than 100 amino acid residues. Furthermore,
the term is also intended to include proteins, i.e. functional
10 biomolecules comprising at least one polypeptide; when compri-
sing at least two polypeptides, these may form complexes, be
covalently linked, or may be non-covalently linked. The polypep-
tide(s) in a protein can be glycosylated and/or lipidated and/or
comprise prosthetic groups.

15 The term "subsequence" means any consecutive stretch of at least
3 amino acids or, when relevant, of at least 3 nucleotides,
derived directly from a naturally occurring OPGL amino acid
sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general intended
20 to denote an animal species (preferably mammalian), such as *Homo
sapiens*, *Canis domesticus*, etc. and not just one single animal.
However, the term also denotes a population of such an animal
species, since it is important that the individuals immunized
according to the method of the invention all harbour substan-
25 tially the same OPGL allowing for immunization of the animals
with the same immunogen(s). If, for instance, genetic variants
of OPGL exists in different human population it may be necessary
to use different immunogens in these different populations in
order to be able to break the autotolerance towards OPGL in each
30 population.

By the term "*in vivo* down-regulation of OPGL activity" is herein meant reduction in the living organism of the number of interactions between OPGL and its (unknown) receptor (or between OPGL and other possible biologically important binding partners for
5 this molecule). The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in OPGL by antibody binding is the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of OPGL by scaven-
10 ger cells (such as macrophages and other phagocytic cells).

The expression "effecting presentation ... to the immune system" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner. As will appear from the disclosure below, such challenge of the
15 immune system can be effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmaccines" (*i.e.* a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune
20 competent cells in the animal are confronted with the antigen in an immunologically effective manner, whereas the precise mode of achieving this result is of less importance to the inventive idea underlying the present invention.

The term "immunogenically effective amount" has its usual mean-
25 ing in the art, *i.e.* an amount of an immunogen which is capable of inducing an immune response which significantly engages pathogenic agents which share immunological features with the immunogen.

When using the expression that the OPGL has been "modified" is
30 herein meant a chemical modification of the polypeptide which constitutes the backbone of OPGL. Such a modification can e.g. be derivatization (e.g. alkylation) of certain amino acid resi-

dues in the OPGL sequence, but as will be appreciated from the
disclosure below, the preferred modifications comprise changes
of the primary structure of the OPGL amino acid sequence.

When discussing "autotolerance towards OPGL" is understood that
5 since OPGL is a self-protein in the population to be vaccinated,
normal individuals in the population do not mount an immune
response against OPGL; it cannot be excluded, though, that
occasional individuals in an animal population might be able to
produce antibodies against native OPGL, e.g. as part of a auto-
10 immune disorder. At any rate, an animal will normally only be
autotolerant towards its own OPGL, but it cannot be excluded
that OPGL analogues derived from other animal species or from a
population having a different OPGL phenotype would also be
tolerated by said animal.

15 A "foreign T-cell epitope" is a peptide which is able to bind to
an MHC molecule and stimulates T-cells in an animal species.
Preferred foreign epitopes are "promiscuous" epitopes, i.e.
epitopes which binds to a substantial fraction of MHC molecules
in an animal species or population. Only a very limited number
20 of such promiscuous T-cell epitopes are known, and they will be
discussed in detail below. It should be noted that in order for
the immunogens which are used according to the present invention
to be effective in as large a fraction of an animal population
as possible, it may be necessary to 1) insert several foreign T-
25 cell epitopes in the same OPGL analogue or 2) prepare several
OPGL analogues wherein each analogue has a different promiscuous
epitope inserted. It should be noted that the concept of foreign
T-cell epitopes also encompasses use of cryptic T-cell epitopes,
i.e. epitopes which are derived from a self-protein and which
30 only exerts immunogenic behaviour when existing in isolated form
without being part of the self-protein in question.

A "functional part" of a (bio)molecule is in the present context
intended to mean the part of the molecule which is responsible
for at least one of the biochemical or physiological effects
exerted by the molecule. It is well-known in the art that many
5 enzymes and other effector molecules have an active site which
is responsible for the effects exerted by the molecule in ques-
tion. Other parts of the molecule may serve a stabilizing or
solubility enhancing purpose and can therefore be left out if
these purposes are not of relevance in the context of a certain
10 embodiment of the present invention. For instance it is possible
to use certain cytokines as a modifying moiety in OPGL (cf. the
detailed discussion below), and in such a case, the issue of
stability may be irrelevant since the coupling to OPGL provides
the stability necessary.

15 Preferred embodiments of OPGL activity down-regulation

It is preferred that the OPGL polypeptide used as an immunogen
in the method of the invention is a modified molecule, since the
chances of obtaining the all-important breaking of autotolerance
towards OPGL is greatly facilitated that way. It should be noted
20 that this does not exclude the possibility of using such a
modified OPGL in formulations which further facilitate the
breaking of autotolerance against OPGL, e.g. formulations con-
taining adjuvants.

It has been shown (in Dalum I et al., 1996, J. Immunol. 157:
25 4796-4804) that potentially self-reactive B-lymphocytes recog-
nizing self-proteins are physiologically present in normal
individuals. However, in order for these B-lymphocytes to be
induced to actually produce antibodies reactive with the rele-
vant self-proteins, assistance is needed from cytokine producing
30 T-helper lymphocytes (T_H-cells or T_H-lymphocytes). Normally this
help is not provided because T-lymphocytes in general do not
recognize T-cell epitopes derived from self-proteins when pre-

sented by antigen presenting cells (APCs). However, by providing
an element of "foreignness" in a self-protein (i.e. by introdu-
cing an immunologically significant modification), T-cells
recognizing the foreign element are activated upon recognizing
5 the foreign epitope on an APC (such as, initially, a mononuclear
cell). Polyclonal B-lymphocytes (which are also APCs) capable of
recognising self-epitopes on the modified self-protein also
internalise the antigen and subsequently presents the foreign T-
cell epitope(s) thereof, and the activated T-lymphocytes subse-
10 quently provide cytokine help to these self-reactive polyclonal
B-lymphocytes. Since the antibodies produced by these polyclonal
B-lymphocytes are reactive with different epitopes on the modi-
fied polypeptide, including those which are also present in the
native polypeptide, an antibody cross-reactive with the non-
15 modified self-protein is induced. In conclusion, the T-lympho-
cytes can be led to act as if the population of polyclonal
B-lymphocytes have recognised an entirely foreign antigen,
whereas in fact only the inserted epitope(s) is/are foreign to
the host. In this way, antibodies capable of *cross-reacting* with
20 non-modified self-antigens are induced.

Several ways of modifying a peptide self-antigen in order to
obtain breaking of autotolerance are known in the art. Hence,
according to the invention, the modification can include that

-    at least one foreign T-cell epitope is introduced, and/or
25 -   at least one first moiety is introduced which effects
     targeting of the modified molecule to an antigen presenting
     cell (APC), and/or
-    at least one second moiety is introduced which stimulates
     the immune system, and/or
30 -   at least one third moiety is introduced which optimizes
     presentation of the modified OPGL polypeptide to the immune
     system.

However, all these modifications should be carried out while maintaining a substantial fraction of the original B-lymphocyte epitopes in OPGL, since the B-lymphocyte recognition of the native molecule is thereby enhanced.

5 In one preferred embodiment, side groups (in the form of foreign T-cell epitopes or the above-mentioned first, second and third moieties) are covalently or non-covalently introduced. This is to mean that stretches of amino acid residues derived from OPGL are derivatized without altering the primary amino acid sequen-
10 ce, or at least without introducing changes in the peptide bonds between the individual amino acids in the chain.

An alternative, and preferred, embodiment utilises amino acid substitution and/or deletion and/or insertion and/or addition (which may be effected by recombinant means or by means of
15 peptide synthesis; modifications which involves longer stretches of amino acids can give rise to fusion polypeptides). One espe- cially preferred version of this embodiment is the technique described in WO 95/05849, which discloses a method for down- regulating self-proteins by immunising with analogues of the
20 self-proteins wherein a number of amino acid sequence(s) has been substituted with a corresponding number of amino acid sequence(s) which each comprise a foreign immunodominant T-cell epitope, while at the same time maintaining the overall tertiary structure of the self-protein in the analogue. For the purposes
25 of the present invention, it is however sufficient if the modi- fication (be it an insertion, addition, deletion or substitu- tion) gives rise to a foreign T-cell epitope and at the same time preserves a substantial number of the B-cell epitopes in OPGL. However, in order to obtain maximum efficacy of the immune
30 response induced, it is preferred that the overall tertiary structure of OPGL is maintained in the modified molecule.

The following formula describes the OPGL constructs generally covered by the invention:

$$(MOD_1)_{s1}(OPGL_{e1})_{n1}(MOD_2)_{s2}(OPGL_{e2})_{n2}\ldots(MOD_x)_{sx}(OPGL_{ex})_{nx} \quad (I)$$

-where $OPGL_{e1}$-$OPGL_{ex}$ are x B-cell epitope containing subsequences

5 of OPGL which independently are identical or non-identical and which may contain or not contain foreign side groups, x is an integer $\geq$ 3, n1-nx are x integers $\geq$ 0 (at least one is $\geq$ 1), MOD_1-MOD_x are x modifications introduced between the preserved B-cell epitopes, and s_1-s_x are x integers $\geq$ 0 (at least one is $\geq$ 1

10 if no side groups are introduced in the $OPGL_e$ sequences). Thus, given the general functional restraints on the immunogenicity of the constructs, the invention allows for all kinds of permu-tations of the original OPGL sequence, and all kinds of modifi-cations therein. Thus, included in the invention are modified

15 OPGL obtained by omission of parts of the OPGL sequence which e.g. exhibit adverse effects *in vivo* or omission of parts which are normally intracellular and thus could give rise to undesired immunological reactions.

20 Maintenance of a substantial fraction of B-cell epitopes or even the overall tertiary structure of a protein which is subjected to modification as described herein can be achieved in several ways. One is simply to prepare a polyclonal antiserum directed against OPGL (e.g. an antiserum prepared in a rabbit) and there-

25 after use this antiserum as a test reagent (e.g. in a competi-tive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does OPGL must be regarded as having the same overall tertiary structure as OPGL whereas analogues

30 exhibiting a limited (but still significant and specific) reac-tivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive with distinct epitopes on OPGL can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of OPGL and 2) a mapping of the epitopes which
5 are maintained in the analogues prepared.

Of course, a third approach would be to resolve the 3-dimensional structure of OPGL or of a biologically active truncate thereof (cf. above) and compare this to the resolved three-dimensional structure of the analogues prepared. Three-dimen-
10 sional structure can be resolved by the aid of X-ray diffraction studies, circular dichroism studies, NMR-spectroscopy etc. Of these, circular dichroism has the advantage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide and NMR requires the
15 provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule. However, ultimately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism only provides indirect evidence of correct 3-dimensional struc-
20 ture via information of secondary structure elements.

One preferred embodiment of the invention utilises multiple presentations of B-lymphocyte epitopes of OPGL (i.e. formula I wherein at least one B-cell epitope is present in two positions). This effect can be achieved in various ways, e.g. by
25 simply preparing fusion polypeptides comprising the structure $(OPGL)_m$, where m is an integer $\geq$ 2 and then introduce the modifications discussed herein in at least one of the OPGL sequences. It is preferred that the modifications introduced includes at least one duplication of a B-lymphocyte epitope and/or the
30 introduction of a hapten.

As mentioned above, the introduction of a foreign T-cell epitope can be accomplished by introduction of at least one amino acid

insertion, addition, deletion, or substitution. Of course, the normal situation will be the introduction of more than one change in the amino acid sequence (e.g. insertion of or substition by a complete T-cell epitope) but the important goal
5 to reach is that the OPGL analogue, when processed by an antigen presenting cell (APC), will give rise to such a foreign immunodominant T-cell epitope being presented in context of an MCH Class II molecule on the surface of the APC. It is preferred that the number of amino acid insertions, deletions, substitu-
10 tions or additions is at least 2, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 25 insertions, substitutions, additions or deletions. It is furthermore preferred that the number of amino acid substitutions is not in excess of 150, such as at most 100, at most 90, at most 80, and
15 at most 70. It is especially preferred that the number of substitutions, insertions, deletions, or additions does not exceed 60, and in particular the number should not exceed 50 or even 40. Most preferred is a number of not more than 30.

Preferred embodiments of the invention includes modification by
20 introducing at least one foreign immunodominant T-cell epitope. It will be understood that the question of immune dominance of a T-cell epitope depends on the animal species in question. As used herein, the term "immunodominance" simply refers to epitopes which in the vaccinated individual gives rise to a signifi-
25 cant immune response, but it is a well-known fact that a T-cell epitope which is immunodominant in one individual is not necessarily immunodominant in another individual of the same species, even though it may be capable of binding MHC-II molecules in the latter individual.

30 Another important point is the issue of MHC restriction of T-cell epitopes. In general, naturally occurring T-cell epitopes are MHC restricted, *i.e.* a certain peptides constituting a T-cell epitope will only bind effectively to a subset of MHC Class

II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be

5 necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are OPGL variants which are distinguished from each other by the nature of the T-cell epitope introduced.

If the MHC restriction of the T-cells used is completely unknown
10 (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

15
$$f_{population} = 1 - \prod_{i=1}^{n} (1 - p_i) \qquad (II)$$

-where p_i is the frequency in the population of responders to the ith foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing
20 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-i.e. 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

25 The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and DR7, then the use of this peptide together with another peptide

which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the coverage

5 at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 \qquad (III)$$

10 -wherein φ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the jth of the 3 known HLA loci (DP, DR and DQ); in practice, it is first determined which MHC molecules will recognize each T-cell epito-

15 pe in the vaccine and thereafter these are listed by type (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding φ_1, φ_2, and φ_3.

It may occur that the value p_i in formula II exceeds the corre-
20 sponding theoretical value π_i:

$$\pi_i = 1 - \prod_{j=1}^{3} (1 - v_j)^2 \qquad (IV)$$

-wherein v_j is the sum of frequencies in the population of allelic haplotype encoding MHC molecules which bind the ith T-cell epitope in the vaccine and which belong to the jth of the 3

25 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of the population is a frequency of responders of $f_{residual\_i} = (p_i - \pi_i)/(1-$

n_i). Therefore, formula III can be adjusted so as to yield formula V:

$$f_{population} = 1 - \prod_{j=1}^{3}(1-\varphi_j)^2 + \left(1 - \prod_{i=1}^{n}(1-f_{residual\_i})\right)$$ (V)

−where the term $1-f_{residual-i}$ is set to zero if negative. It should
5 be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the OPGL analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders
10 in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are
15 preferably introduced in the vaccine thereby reducing the need for a very large number of different OPGL analogues in the same vaccine.

The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from
20 tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxo-id, Influenza virus hemagluttinin (HA), and *P. falciparum* CS antigen.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a
25 large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible T-cell epitopes to be introduced in the OPGL analogues used according to the present invention. Cf. also the epitopes dis-

cussed in the following references which are hereby all incorpo-
rated by reference herein: Southwood S *et. al*, 1998, J. Immunol.
160: 3363-3373; Sinigaglia F *et al.*, 1988, Nature **336**: 778-780;
Chicz RM *et al.*, 1993, J. Exp. Med **178**: 27-47; Hammer J *et al.*,
5 1993, Cell **74**: 197-203; and Falk K *et al.*, 1994, Immunogenetics
39: 230-242. The latter reference also deals with HLA-DQ and -DP
ligands. All epitopes listed in these 5 references are relevant
as candidate natural epitopes to be used in the present inven-
tion, as are epitopes which share common motifs with these.

10 Alternatively, the epitope can be any artificial T-cell epitope
which is capable of binding a large proportion of haplotypes. In
this context the pan DR epitope peptides ("PADRE") described in
WO 95/07707 and in the corresponding paper Alexander J *et al.*,
1994, Immunity **1**: 751-761 (both disclosures are incorporated by
15 reference herein) are interesting candidates for epitopes to be
used according to the present invention. It should be noted that
the most effective PADRE peptides disclosed in these papers
carry D-amino acids in the C- and N-termini in order to improve
stability when administered. However, the present invention
20 primarily aims at incorporating the relevant epitopes as part of
the modified OPGL which should then subsequently be broken down
enzymatically inside the lysosomal compartment of APCs to allow
subsequent presentation in the context of an MHC-II molecule and
therefore it is not expedient to incorporate D-amino acids in
25 the epitopes used in the present invention.

One especially preferred PADRE peptide is the one having the
amino acid sequence AKFVAAWTLKAAA or an immunologically effec-
tive subsequence thereof. This, and other epitopes having the
same lack of MHC restriction are preferred T-cell epitopes which
30 should be present in the OPGL analogues used in the inventive
method. Such super-promiscuous epitopes will allow for the most
simple embodiments of the invention wherein only one single

modified OPGL is presented to the vaccinated animal's immune
system.

As mentioned above, the modification of OPGL can also include
the introduction of a first moiety which targets the modified
5 OPGL to an APC or a B-lymphocyte. For instance, the first moiety
can be a specific binding partner for a B-lymphocyte specific
surface antigen or for an APC specific surface antigen. Many
such specific surface antigens are known in the art. For in-
stance, the moiety can be a carbohydrate for which there is a
10 receptor on the B-lymphocyte or the APC (e.g. mannan or man-
nose). Alternatively, the second moiety can be a hapten. Also an
antibody fragment which specifically recognizes a surface mole-
cule on APCs or lymphocytes can be used as a first moiety (the
surface molecule can e.g. be an FCγ receptor of macrophages and
15 monocytes, such as FCγRI or, alternatively any other specific
surface marker such as CD40 or CTLA-4). It should be noted that
all these exemplary targeting molecules can be used as part of
an adjuvant, cf. below.

As an alternative or supplement to targeting the modified OPGL
20 polypeptide to a certain cell type in order to achieve an en-
hanced immune response, it is possible to increase the level of
responsiveness of the immune system by including the above-
mentioned second moiety which stimulates the immune system.
Typical examples of such second moieties are cytokines, and
25 heat-shock proteins, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are
those which will normally also function as adjuvants in a vac-
cine composition, i.e. for instance interferon γ (IFN-γ),
interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-
30 4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13
(IL-13), interleukin 15 (IL-15), and granulocyte-macrophage
colony stimulating factor (GM-CSF); alternatively, the func-

tional part of the cytokine molecule may suffice as the second moiety. With respect to the use of such cytokines as adjuvant substances, cf. the discussion below.

According to the invention, suitable heat shock proteins used as
5 the second moiety can be HSP70, HSP90, and HSC70.

Alternatively, the second moiety can be a toxin, such as listeriolycin (LLO), lipid A and heat-labile enterotoxin. Also, a number of mycobacterial derivatives such as MDP (muramyl dipeptide), CFA (complete Freund's adjuvant) and the trehalose
10 diesters TDM and TDE are interesting possibilities.

Also the possibility of introducing a third moiety which en-
hances the presentation of the modified OPGL to the immune system is an important embodiment of the invention. The art has shown several examples of this principle. For instance, it is
15 known that the palmitoyl lipidation anchor in the *Borrelia burgdorferi* protein OspA can be utilised so as to provide self-
adjuvating polypeptides (cf. e.g. WO 96/40718) It seems that the lipidated proteins form up micelle-like structures with a core consisting of the lipidation anchor parts of the polypeptides
20 and the remaining parts of the molecule protruding therefrom, resulting in multiple presentations of the antigenic determi-
nants. Hence, the use of this and related approaches using different lipidation anchors (e.g. a myristyl anchor) are pre-
ferred embodiments of the invention, especially since the provi-
25 sion of such a lipidation anchor in a recombinantly produced protein is fairly straightforward and merely requires use of e.g. a naturally occurring signal sequence as a fusion partner for the modified OPGL polypeptide. Another possibility is use of the C3d fragment of complement factor C3 or C3 itself (cf.
30 Dempsey *et al.*, 1996, Science **271**, 348-350 and Lou & Kohler, 1998, Nature Biotechnology **16**, 458-462).

An alternative embodiment of the invention which also results in
the preferred presentation of multiple (e.g. at least 2) copies
of the important epitopic regions of OPGL to the immune system
is the covalent coupling of OPGL, subsequence or variants there-
5 of to certain molecules. For instance, polymers can be used,
e.g. carbohydrates such as dextran, cf. e.g. Lees A et al.,
1994, Vaccine **12**: 1160-1166; Lees A et al., 1990, J Immunol.
145: 3594-3600, but also mannose and mannan are useful alterna-
tive. Integral membrane proteins from e.g. *E. coli* and other
10 bacteria are also useful conjugation partners. The traditional
carrier molecules such as keyhole limpet hemocyanin (KLH),
tetanus toxoid, diphtheria toxoid, and bovine serum albumin
(BSA) are also preferred and useful conjugation partners.

Certain areas of native OPGL seems to be most suited for per-
15 forming modifications. Because of OPGL's structural relationship
with TNF-α and other members of the tumour necrosis factor
family, it is predicted that introductions of T-cell epitopes in
areas defined by positions 170-193 and/or 198-219 and/or 222-247
and/or 285-317 will be most likely to produce the desired re-
20 sults. Considerations underlying these chosen areas are a)
preservation of known and predicted B-cell epitopes, b) preser-
vation of tertiary structure etc. At any rate, as discussed
above, it is fairly easy to screen a library of modified OPGL
molecules which have all been subjected to introduction of a T-
25 cell epitope in different locations.

Formulation of OPGL and modified OPGL polypeptides

When effecting presentation of the OPGL polypeptide or the
modified OPGL polypeptide to an animal's immune system by means
of administration thereof to the animal, the formulation of the
30 polypeptide follows the principles generally acknowledged in the
art.

Preparation of vaccines which contain peptide sequences as
active ingredients is generally well understood in the art, as
exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231;
4,599,230; 4,596,792; and 4,578,770, all incorporated herein by
5 reference. Typically, such vaccines are prepared as injectables
either as liquid solutions or suspensions; solid forms suitable
for solution in, or suspension in, liquid prior to injection may
also be prepared. The preparation may also be emulsified. The
active immunogenic ingredient is often mixed with excipients
10 which are pharmaceutically acceptable and compatible with the
active ingredient. Suitable excipients are, for example, water,
saline, dextrose, glycerol, ethanol, or the like, and combina-
tions thereof. In addition, if desired, the vaccine may contain
minor amounts of auxiliary substances such as wetting or emulsi-
15 fying agents, pH buffering agents, or adjuvants which enhance
the effectiveness of the vaccines; cf. the detailed discussion
of adjuvants below.

The vaccines are conventionally administered parenterally, by
injection, for example, either subcutaneously, intradermally,
20 subdermally or intramuscularly. Additional formulations which
are suitable for other modes of administration include supposi-
tories and, in some cases, oral, buccal, sublinqual,
intraperitoneal, intravaginal, anal and intracranial formula-
tions. For suppositories, traditional binders and carriers may
25 include, for example, polyalkalene glycols or triglycerides;
such suppositories may be formed from mixtures containing the
active ingredient in the range of 0.5% to 10%, preferably 1-2%.
Oral formulations include such normally employed excipients as,
for example, pharmaceutical grades of mannitol, lactose, starch,
30 magnesium stearate, sodium saccharine, cellulose, magnesium
carbonate, and the like. These compositions take the form of
solutions, suspensions, tablets, pills, capsules, sustained
release formulations or powders and contain 10-95% of active
ingredient, preferably 25-70%. For oral formulations, cholera

toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid

5 addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from

10 inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the

15 dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage

20 ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 µg to 2000 µg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 1000 µg, preferably in the range from 1 µg to 500 µg and espe-

25 cially in the range from about 10 µg to 100 µg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the

30 conventional methods for administration of a vaccine are applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion,

parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and the formulation of the antigen.

5 Some of the polypeptides of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance. It is especially preferred to use an adjuvant which can be demonstrated to facilitate breaking of the

10 autotolerance to autoantigens; in fact, this is essential in cases where unmodified OPGL is used as the active ingredient in the autovaccine.

Various methods of achieving adjuvant effect for the vaccine are known. General principles and methods are detailed in "The

15 Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generationn Immunological Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press, New York, ISBN 0-306-45283-9, both of which are hereby incorporated

20 by reference herein.

The application of adjuvants include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent

25 solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to

30 albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles

such as mannide mono-oleate (Aracel A) or emulsion with 20
percent solution of a perfluorocarbon (Fluosol-DA) used as a
block substitute may also be employed. Admixture with oils such
as squalene and IFA is also preferred.

5 According to the invention DDA (dimethyldioctadecylammonium
bromide) is an interesting candidate for an adjuvant as is DNA
and γ-inulin, but also Freund's complete and incomplete adju-
vants as well as *quillaja* saponins such as QuilA and QS21 are
interesting as is RIBI. Further possibilities are monophosphoryl
10 lipid A (MPL), the above mentioned C3 and C3d, and muramyl
dipeptide (MDP).

Liposome formulations are also known to confer adjuvant effects,
and therefore liposome adjuvants are preferred according to the
invention.

15 Also immunostimulating complex matrix type (ISCOM® matrix)
adjuvants are preferred choices according to the invention,
especially since it has been shown that this type of adjuvants
are capable of up-regulating MHC Class II expression by APCs. An
ISCOM® matrix consists of (optionally fractionated) saponins
20 (triterpenoids) from *Quillaja saponaria*, cholesterol, and
phospholipid. When admixed with the immunogenic protein, the
resulting particulate formulation is what is known as an ISCOM
particle where the saponin constitutes 60-70% w/w, the choles-
terol and phospholipid 10-15% w/w, and the protein 10-15% w/w.
25 Details relating to composition and use of immunostimulating
complexes can e.g. be found in the above-mentioned text-books
dealing with adjuvants, but also Morein B et al., 1995, Clin.
Immunother. 3: 461-475 as well as Barr IG and Mitchell GF, 1996,
Immunol. and Cell Biol. 74: 8-25 (both incorporated by reference
30 herein) provide useful instructions for the preparation of
complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin et al., 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant antigen

5 such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen binding antibody fragments) against the Fcγ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-FcγRI have been demonstrated to enhance immunogenicity for the pur-

10 poses of vaccination.

Other possibilities involve the use of the targeting and immune modulating substances (i.a. cytokines) mentioned above as candidates for the first and second moieties in the modified versions of OPGL. In this connection, also synthetic inducers of cyto-

15 kines like poly I:C are possibilities.

In general it should be noted that the disclosures above which relate to compounds and agents useful as first, second and third moieties in the modified OPGL also refer mutatis mutandis to their use in the adjuvant of a vaccine of the invention.

20 It is expected that the vaccine should be administered 1-6 times per year, such as 1, 2, 3, 4, 5, or 6 times a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred autovaccines according to the invention is not permanent, and therefor the

25 immune system needs to be periodically challenged with the OPGL or modified OPGL polypeptides.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise

30 several different polypeptides in order to increase the immune response, cf. also the discussion above concerning the choice of

foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above.

The vaccine may consequently comprise 3-20 different modified or
5 unmodified polypeptides, such as 3-10 different polypeptides.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation", and
10 "gene immunisation") offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the form of indus- trial scale fermentation of microorganisms producing modified
15 OPGL). Furthermore, there is no need to device purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational
20 processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant fraction of the original OPGL B-cell epitopes should be preserved in the modified molecule, and since B-cell epitopes in principle can be constituted by
25 parts of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is best ensured by having the host producing the immunogen.

Hence, a preferred embodiment of the invention comprises effect-
ing presentation of modified OPGL to the immune system by intro-
ducing nucleic acid(s) encoding the modified OPGL into the
animal's cells and thereby obtaining *in vivo* expression by the
5 cells of the nucleic acid(s) introduced.

In this embodiment, the introduced nucleic acid is preferably
DNA which can be in the form of naked DNA, DNA formulated with
charged or uncharged lipids, DNA formulated in liposomes, DNA
included in a viral vector, DNA formulated with a transfection-
10 facilitating protein or polypeptide, DNA formulated with a
targeting protein or polypeptide, DNA formulated with Calcium
precipitating agents, DNA coupled to an inert carrier molecule,
and DNA formulated with an adjuvant. In this context it is noted
that practically all considerations pertaining to the use of
15 adjuvants in traditional vaccine formulation apply for the
formulation of DNA vaccines. Hence, all disclosures herein which
relate to use of adjuvants in the context of polypeptide based
vaccines apply *mutatis mutandis* to their use in nucleic acid
vaccination technology.

20 Furthermore, the nucleic acid used as an immunization agent can
contain regions encoding the immunomodulating substances de-
scribed above, e.g. in the form of the cytokines discussed as
useful adjuvants. A preferred version of this embodiment encom-
passes having the coding region for the modified OPGL and the
25 coding region for the immunomodulator in different reading
frames or at least under the control of different promoters.
Thereby it is avoided that the modified OPGL is produced as a
fusion partner to the immunomodulator. Alternatively, two dis-
tinct nucleotide fragments can be used, but this is less
30 preffered because of the advantage of ensured co-expression when
having both coding regions included in the same molecule.

Accordingly, the invention also relates to a composition for inducing production of antibodies against OPGL, the composition comprising

- a nucleic acid fragment or a vector of the invention (cf.

5   the discussion of vectors below), and

- a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and/or adjuvant as discussed above.

Under normal circumstances, the OPGL variant-encoding nucleic

10 acid is introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al,

15 1997, Annu. Rev. Immunol. **15**: 617-648 and Donnelly JJ et al., 1997, Life Sciences **60**: 163-172. Both of these references are incorporated by reference herein.

Live vaccines

A third alternative for effecting presentation of modified OPGL

20 to the immune system is the use of live vaccine technology. In live vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic microorganism which has been transformed with a nucleic acid fragment encoding a modified OPGL or with a vector incorporating such a nucleic

25 acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella*

30 spp., *Vibrio cholerae*, *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. **45**: 1492-1496 and Walker PD, 1992,

Vaccine 10: 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live vaccines, cf. the discussion below.

As an alternative to bacterial live vaccines, the nucleic acid
5 fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector such as a vaccinia strain.

Use of the method of the invention in disease treatment

As will be appreciated from the discussions above, the provision of the method of the invention allows for control of diseases
10 characterized by excessive loss of bone mass. In this context, the disease osteoporosis is the key target for the inventive method but also bone loss associated with complicated bone fractures is a feasible target for treatment/amelioration. Hence, an important embodiment of the method of the invention
15 for down-regulating OPGL activity comprises treating and/or preventing and/or ameliorating osteoporosis or other conditions characterized by excess bone resorption, the method comprising down-regulating OPGL activity according to the method of the invention to such an extent that the rate of bone resorption is
20 significantly decreased.

In the present context such a significant decrease in bone resorption is at least 3% compared to the pathological rate, but higher percentages are contemplated, such as at least 5%, at least 7%, at least 9%, at least 11%, at least 13%, at least 15%,
25 and at least 17%, but even higher percentages are expected, such as at least 20%, or even at least 30%. It is especially pre- ferred that the decrease in bone resorption results in an inver- sion of the balance between bone formation and bone resorption, i.e. that the rate of bone formation is brought to exceed the
30 rate of bone resorption. Of course, this imbalance should not be maintained (since it would result in osteopetrosis), but by

carefully controlling the number of immunizations of the individual in need thereof it is possible to obtain a balance over time which results in a net conservation of bone mass. Alternatively, if in an individual the method of the invention cannot

5 terminate bone loss, the method of the invention can (optionally in combination with other known methods for reducing bone loss in osteoporosis patients) be used to obtain a significant reduction in bone loss, thereby prolonging the time where sufficient bone mass is present in the individual.

10 Methods for measuring the rate of bone resorption and bone formation are known in the art. It is by means of biochemical assays possible to gauge the rate of bone resorption by measuring the blood concentration of certain fragments of collagen type I (cf. WO 93/15107 and WO 94/14844). Alternatively, the

15 rate of bone loss can be assessed by physical means; representative disclosures in the art of methods for assessing bone mass by non-invasive, physical methods can be found in WO 88/06862, WO 94/12855, WO 95/14431, and WO 97/00643.

Peptides, polypeptides, and compositions of the invention

20 As will be apparent from the above, the present invention is based on the concept of immunising individuals against the OPGL antigen in order to indirectly obtain a reduced osteoclast activity. The preferred way of obtaining such an immunization is to use modified versions of OPGL, thereby providing molecules

25 which have not previously been disclosed in the art.

It is believed that the modified OPGL molecules discussed herein are inventive in their own right, and therefore an important part of the invention pertains to an OPGL analogue which is derived from an animal OPGL wherein is introduced a modification

30 which has as a result that immunization of the animal with the analogue induces production of antibodies reacting specifically

with the unmodified OPGL polypeptide. Preferably, the nature of the modification conforms with the types of modifications described above when discussing various embodiments of the method of the invention when using modified OPGL. Hence, any disclosure
5 presented herein pertaining to modified OPGL molecules are relevant for the purpose of describing the OPGL analogues of the invention, and any such disclosures apply *mutatis mutandis* to the description of these analogues.

It should be noted that preferred modified OPGL molecules com-
10 prises modifications which results in a polypeptide having a sequence identity of at least 70% with OPGL or with a subsequence thereof of at least 10 amino acids in length. Higher sequence identities are preferred, e.g. at least 75% or even at least 80% or 85%. The sequence identity for proteins and nucleic acids can
15 be calculated as $\left(N_{ref} - N_{dif}\right) \cdot 100/N_{ref}$, wherein N_{dif} is the total

number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N_{dif}=2 and N_{ref}=8).

20 The invention also pertains to compositions useful in exercising the method of the invention. Hence, the invention also relates to an immunogenic composition comprising an immunogenically effective amount of an OPGL polypeptide which is a self-protein in an animal, said OPGL polypeptide being formulated together
25 with an immunologically acceptable adjuvant so as to break the animal's autotolerance towards the OPGL polypeptide, the composition further comprising a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient. In other words, this part of the invention pertains to the
30 formulations of naturally occurring OPGL polypeptides which have

been described in connection with embodiments of the method of
the invention.

The invention also relates to an immunogenic composition com-
prising an immunologically effective amount of an OPGL analogue
5 defined above, said composition further comprising a pharmaceu-
tically and immunologically acceptable diluent and/or vehicle
and/or carrier and/or excipient and optionally an adjuvant. In
other words, this part of the invention concerns formulations of
modified OPGL, essentially as described hereinabove. The choice
10 of adjuvants, carriers, vehicle, excipients, and diluents is
accordingly in line with what has been discussed above when
referring to formulation of modified and unmodified OPGL for use
in the inventive method for the down-regulation of OPGL.

The polypeptides are prepared according to methods well-known in
15 the art. Longer polypeptides are normally prepared by means of
recombinant gene technology including introduction of a nucleic
acid sequence encoding the OPGL analogue into a suitable vector,
transformation of a suitable host cell with the vector, expres-
sion of the nucleic acid sequence, recovery of the expression
20 product from the host cells or their culture supernatant, and
subseqeunt purification and optional further modification, e.g.
refolding or derivatization.

Shorter peptides are preferably prepared by means of the well-
known techniques of solid- or liquid-phase peptide synthesis.
25 However, recent advances in this technology has rendered possi-
ble the production of full-length polypeptides and proteins by
these means, and therefore it is also within the scope of the
present invention to prepare the long constructs by synthetic
means.

Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that modified
OPGL polypeptides can be prepared by means of recombinant gene
technology but also by means of chemical synthesis or
5 semisynthesis; the latter two options are especially relevant
when the modification consists in coupling to protein carriers
(such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and
non-proteinaceous molecules such as carbohydrate polymers and of
course also when the modification comprises addition of side
10 chains or side groups to an OPGL polypeptide-derived peptide
chain.

For the purpose of recombinant gene technology, and of course
also for the purpose of nucleic acid immunization, nucleic acid
fragments encoding modified OPGL are important chemical pro-
15 ducts. Hence, an important part of the invention pertains to a
nucleic acid fragment which encodes an OPGL analogue, i.e. an
OPGL derived polypeptide which either comprises the natural OPGL
sequence to which has been added or inserted a fusion partner
or, preferably an OPGL derived polypeptide wherein has been
20 introduced a foreign T-cell epitope by means of insertion and/or
addition, preferably by means of substitution and/or deletion.
The nucleic acid fragments of the invention are either DNA or
RNA fragments.

The nucleic acid fragments of the invention will normally be
25 inserted in suitable vectors to form cloning or expression
vectors carrying the nucleic acid fragments of the invention;
such novel vectors are also part of the invention. Details
concerning the construction of these vectors of the invention
will be discussed in context of transformed cells and microor-
30 ganisms below. The vectors can, depending on purpose and type of
application, be in the form of plasmids, phages, cosmids, mini-

chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector. Preferred cloning and expression vectors of the invention are capable of autonomous replication, thereby enabling high copy-numbers
5 for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the following features in the 5'→3' direction and in operable linkage: a promoter for driving expression of the nucleic acid
10 fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment of the invention, and a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer
15 strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the vector when introduced into a host cell is integrated in the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an animal (*i.e.* when using the
20 vector in DNA vaccination) it is for security reasons preferred that the vector is not incapable of being integrated in the host cell genome; typically, naked DNA or non-integrating viral vectors are used, the choices of which are well-known to the *person skilled in the art*.

25 The vectors of the invention are used to transform host cells to produce the modified OPGL polypeptide of the invention. Such transformed cells, which are also part of the invention, can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recom-
30 binant production of the modified OPGL polypeptides of the invention. Alternatively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid fragment (one single or multiple copies) have been inserted so as to effect

secretion or integration into the bacterial membrane or cell-wall of the modified OPGL.

Preferred transformed cells of the invention are microorganisms such as bacteria (such as the species *Escherichia* [e.g. *E.coli*],
5 *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or *Mycobacterium* [preferably non-pathogenic, e.g. *M. bovis* BCG]), yeasts (such as *Saccharomyces cerevisiae*), and protozoans. Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect cell, a plant cell, or a mammalian cell.
10 Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below.

For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing the
15 nucleic fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the modified OPGL or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

When producing the modified OPGL of the invention by means of
20 transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.

When an effective producer cell has been identified it is pre-
25 ferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the modified OPGL. Preferably, this stable cell line secretes or carries the OPGL analogue of the invention, thereby facilitating purification there-
30 of.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector ordinarily carries a replication site, as well as marking se-
5 quences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides
10 easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.

Those promoters most commonly used in recombinant DNA construc-
15 tion include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EP-A-0 036 776). While these are the most commonly used, other microbial promoters have been discovered and uti-
20 lized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for
25 addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and here the promoter should be capable of driving expression. *Saccharomyces cerevisiase*, or common baker's yeast is the most commonly used among eukaryotic
30 microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already

contains the trpl gene which provides a selection marker for a
mutant strain of yeast lacking the ability to grow in tryptophan
for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence
of the trpl lesion as a characteristic of the yeast host cell
5 genome then provides an effective environment for detecting
transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the pro-
moters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or
other glycolytic enzymes (Hess et al., 1968; Holland et al.,
10 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogena-
se, hexokinase, pyruvate decarboxylase, phosphofructokinase,
glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyru-
vate kinase, triosephosphate isomerase, phosphoglucose isome-
rase, and glucokinase. In constructing suitable expression
15 plasmids, the termination sequences associated with these genes
are also ligated into the expression vector 3' of the sequence
desired to be expressed to provide polyadenylation of the mRNA
and termination.

Other promoters, which have the additional advantage of tran-
20 scription controlled by growth conditions are the promoter
region for alcohol dehydrogenase 2, isocytochrome C, acid phos-
phatase, degradative enzymes associated with nitrogen metabo-
lism, and the aforementioned glyceraldehyde-3-phosphate
dehydrogenase, and enzymes responsible for maltose and galactose
25 utilization. Any plasmid vector containing a yeast-compatible
promoter, origin of replication and termination sequences is
suitable.

In addition to microorganisms, cultures of cells derived from
multicellular organisms may also be used as hosts. In principle,
30 any such cell culture is workable, whether from vertebrate or
invertebrate culture. However, interest has been greatest in
vertebrate cells, and propagation of vertebrate in culture

(tissue culture) has become a routine procedure in recent years
(Tissue Culture, 1973). Examples of such useful host cell lines
are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines,
and W138, BHK, COS-7 293 and MDCK cell lines.

5 Expression vectors for such cells ordinarily include (if neces-
sary) an origin of replication, a promoter located in front of
the gene to be expressed, along with any necessary ribosome
binding sites, RNA splice sites, polyadenylation site, and
transcriptional terminator sequences.

10 For use in mammalian cells, the control functions on the expres-
sion vectors are often provided by viral material. For example,
commonly used promoters are derived from polyoma, Adenovirus 2,
and most frequently Simian Virus 40 (SV40). The early and late
promoters of SV40 virus are particularly useful because both are
15 obtained easily from the virus as a fragment which also contains
the SV40 viral origin of replication (Fiers et al., 1978).
Smaller or larger SV40 fragments may also be used, provided
there is included the approximately 250 bp sequence extending
from the HindIII site toward the BglI site located in the viral
20 origin of replication. Further, it is also possible, and often
desirable, to utilize promoter or control sequences normally
associated with the desired gene sequence, provided such control
sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction
25 of the vector to include an exogenous origin, such as may be
derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV,
BPV) or may be provided by the host cell chromosomal replication
mechanism. If the vector is integrated into the host cell chro-
mosome, the latter is often sufficient.

Identification of useful OPGL analogues

It will be clear to the skilled person that not all variants or modifications of native OPGL will have the ability to elicit antibodies in an animal which are cross-reactive with the native

5 form. It is, however, not difficult to set up an effective standard screen for modified OPGL molecules which fulfill the minimum requirements for immunological reactivity discussed herein. Hence, another part of the invention concerns a method for the identification of a modified OPGL polypeptide which is

10 capable of inducing antibodies against unmodified OPGL in an animal species where the unmodified OPGL polypeptide is a self-protein, the method comprising

- preparing, by means of peptide synthesis or by molecular biological means, a library of mutually distinct modified

15 OPGL polypeptides wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of an OPGL polypeptide of the animal species thereby giving rise to amino acid sequences in the library which comprise T-cell epitopes which are and foreign to the

20 animal species,

- testing members of the library for their ability to induce production of antibodies by the animal species against the unmodified OPGL, and

- isolating the member(s) of the library which significantly

25 induces antibody production against unmodified OPGL in the animal species.

In this context, the "library of mutually distinct modified OPGL polypeptides" is a collection of non-identical modified OPGL polypeptides which have e.g. been selected on the basis of the

30 criteria discussed above (e.g. in combination with studies of circular dichroism, NMR spectra, and/or X-ray diffraction pat-

terns). The library may consist of only a few members but it is contemplated that the library may contain several hundred members.

The test of members of the library can ultimately be performed
5 in vivo, but a number of in vitro tests can be applied which narrow down the number of modified molecules which will serve the purpose of the invention.

Since the goal of introducing the foreign T-cell epitopes is to support the B-cell response by T-cell help, a prerequisite is
10 that T-cell proliferation is induced by the modified OPGL. T-cell proliferation can be tested by standardized proliferation assays in vitro. In short, a sample enriched for T-cells is obtained from a subject and subsequently kept in culture. The cultured T-cells are contacted with APCs of the subject which
15 have previously taken up the modified molecule and processed it to present its T-cell epitopes. The proliferation of T-cells is monitored and compared to a suitable control (e.g. T-cells in culture contacted with APCs which have processed intact, native OPGL). Alternatively, proliferation can be measured by determin-
20 ing the concentration of relevant cytokines released by the T-cells in response to their recognition of foreign T-cells.

Having rendered highly probable that at least one modified OPGL of the library is capable of inducing antibody production a-gainst OPGL, it is possible to prepare an immunogenic composi-
25 tion comprising at least one modified OPGL polypeptide which is capable of inducing antibodies against unmodified OPGL in an animal species where the unmodified OPGL polypeptide is a self-protein, the method comprising admixing the member(s) of the library which significantly induces production of antibodies in
30 the animal species which are reactive with OPGL with a pharma-ceutically and immunologically acceptable carrier and/or vehicle and/or diluent and/or excipient, optionally in combination with

at least one pharmaceutically and immunologically acceptable adjuvant.

The above aspects of the invention are conveniently carried out by initially preparing a number of mutually distinct nucleic
5 acid sequences or vectors of the invention, inserting these into appropriate expression vectors, transforming suitable host cells with the vectors, and expressing the nucleic acid sequences of the invention. These steps can be followed by isolation of the expression products. It is preferred that the nucleic acid
10 sequences and/or vectors are prepared by methods comprising exercise of a molecular amplification technique such as PCR or by means of nucleic acid synthesis.

Another part of the invention concerns a method for the treat-
ment, prophylaxis or amelioration of diseases characterized by
15 excess bone resorption in an animal, including a human being, the method comprising administering, to the animal, an effective amount of at least one substance different from osteoprotegerin which blocks the stimulatory effect of OPGL on osteoclast acti-
vity. It is presently believed that such an approach has never
20 been suggested in the art.

The preferred embodiment of this part of the invention involves use of an OPGL-specific antibody (poly- or monoclonal) or a specifically binding variant thereof as the substance blocking the stimulatory effect of OPGL. It is preferred that the anti-
25 body is an IgG or IgM molecule, or that the specifically binding varian is derived from IgG or IgM. The specifically binding variant of the antibody can conveniently be a Fab fragment, a F(ab')$_2$ fragment, a humanized monoclonal antibody or fragment thereof, or a di- or multimeric antibody fragment such as a
30 diabody (a bispecific and dimeric artificial antibody-derived molecule produced by Cambridge Antibody Technology).

EXAMPLE

Initially it is contemplated clone or synthesize cDNAs for murine and human OPGL in the truncated version comprising amino acid residues 158-316 in the murine case and residues 159-317 in

5 the human case (numbers correspond to the numbering in SEQ ID NOs 2, 4 and 6). As these truncated versions have biological activity, it is logical to direct the autoantibodies against this part. In addition, it makes the proteins smaller and thus easier to handle.

10 Expression of these truncated variants of OPGL can take place in *E. coli* as this has already been done (Lacey *et al.*). Alternatively, a yeast expression system could be used if glycosylation is desired. It should be noted that the glycosylation is not needed for biological activity *in vivo* of OPGL. It is also

15 possible to express the truncated OPGL in human 293 fibroblasts as reported in Lacey *et al.*

The purified variants will be used for antibody production in rabbits for later use as detection tools as there exist no commercially available antibodies. In addition, this material

20 will be a very valuable tool in the biological assays needed to evaluate the autovaccine candidates. The preparation of the antibodies will be performed using standard methods known in the art.

The vaccine molecules will initially be constructed by insertion

25 or in-substitution of either the P2 or P30 epitope from tetanus toxoid at selected positions. There is no three dimensional structure known meaning that the selected positions will be chosen based on knowledge of existing or predicted B-cell epitopes and secondary structure elements of the native molecule. The

30 introduction will take place in areas corresponding to amino acid residues 170-193, 198-219, 222-247 and 285-317.

Selecting the best autovaccine candidate is based on assessment of inhibitory activity in *in vitro* assays for osteoclast maturation/activation or in *in vivo* animal models for osteoporosis. Such assays and models are described in the literature (e.g. in

5 Lacey *et al.* and Simonet *et al.*).

It should be noted that as alternative to modified OPGL, antiidiotypic antibodies directed against the idiotype of an anti-OPGL antibody will also serve as useful immunogens within the scope of the present invention. Likewise, the use of mimotypic

10 polypeptides which can be isolated in e.g. a phage display system using anti-OPGL or osteoprotegerin as catching probe are also a part of the invention.

LIST OF REFERENCES

1. Bucay, N. *et al.* (1998), Genes Dev. **12**, 1260-1268.
15 2. Lacey, D. L. *et al.* (1998), Cell **93**, 165-176.
3. Marks, S. C., Jr. (1989), Am. J. Med. Genet. **34**, 43-53.
4. Simonet, W. S. *et al.* (1997), Cell **89**, 309-319.

SEQUENCE LISTING

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65                  70                  75                  80

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Ala Asp Phe Gln Asp Thr Thr Leu Glu Ser Gln Asp Thr Lys Leu Ile
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Glu Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Thr Asp Ile Pro
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Ser Gly Ser His Lys Val Ser Leu Ser Ser Trp Tyr His Asp Arg Gly
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Trp Ala Lys Ile Ser Asn Met Thr Phe Ser Asn Gly Lys Leu Ile Val
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Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
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Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
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Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
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Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
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Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
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Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
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Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
            275             280             285

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Ser Gly Pro Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser
 20                  25                  30                  35

gca ccg gct ccg gcg ccg cca ccc gcc gcc tcc cgc tcc atg ttc ctg   322
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Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser Ile Ala Leu
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Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Gly
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Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys
100                 105                 110                 115
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cac att gtg ggg cca cag cgc ttc tca gga gct cca gct atg atg gaa     610
His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu
            135               140               145

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Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro
            150               155               160

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Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile
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acc agc atc aaa atc cca agt tct cat aac ctg atg aaa gga ggg agc     946
Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser
        245               250               255

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Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn
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            295               300               305

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Gly Ala Phe Lys Val Gln Asp Ile Asp
            310               315
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         35          40              45

Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
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Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
 65              70              75              80

Ser Glu Asp Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu
                 85              90              95

Asn Ala Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro
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Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys
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Met Met Glu Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu
145             150             155             160

Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser
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Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
             180             185             190

Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn
             195             200             205

Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
     210             215             220

Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr
225             230             235             240

Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
                 245             250             255

Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
             260             265             270

Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
             275             280             285
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Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
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Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
305   310   315

CLAIMS

1. A method for *in vivo* down-regulation of osteoprotegerin
ligand (OPGL) activity in an animal, including a human being,
the method comprising effecting presentation to the animal's
5 immune system of an immunologically effective amount of
-       at least one OPGL polypeptide or subsequence thereof which
        has been formulated so that immunization of the animal with
        the OPGL polypeptide or subsequence thereof induces produc-
        tion of antibodies against the OPGL polypeptide, and/or
10 -   at least one OPGL analogue wherein is introduced a modifi-
        cation in the OPGL polypeptide which has as a result that
        immunization of the animal with the analogue induces pro-
        duction of antibodies against the OPGL polypeptide.

2. The method according to claim 1, wherein is presented a
15 modified OPGL polypeptide.

3. The method according to claim 2, wherein the modification has
as a result that a substantial fraction of OPGL B-cell epitopes
are preserved and that
-       at least one foreign T-cell epitope is introduced, and/or
20 -   at least one first moiety is introduced which effects
        targeting of the modified molecule to an antigen presenting
        cell (APC) or a B-lymphocyte, and/or
-       at least one second moiety is introduced which stimulates
        the immune system, and/or
25 -   at least one third moiety is introduced which optimizes
        presentation of the modified OPGL polypeptide to the immune
        system.

4. The method according to claim 3, wherein the modification
30 includes introduction as side groups by covalent or non-covalent
binding to suitable chemical groups in OPGL or a subsequence

thereof of the foreign T-cell epitope and/or the first and/or the second and/or the third moiety.

5. The method according to claim 3 or 4, wherein the modification includes amino acid substitution and/or deletion and/or
5 insertion and/or addition.

6. The method according to claim 5, wherein the modification results in the provision of a fusion polypeptide.

7. The method according to claim 5, wherein introduction of the amino acid substitution and/or deletion and/or insertion and/or
10 addition results in a substantial preservation of the overall tertiary structure of OPGL.

8. The method according to any of claims 2-7, wherein the modification includes duplication of at least one OPGL B-cell epitope and/or introduction of a hapten.

15 9. The method according to any of claims 3-8, wherein the foreign T-cell epitope is immunodominant in the animal.

10. The method according to any of claims 3-9, wherein the foreign T-cell epitope is promiscuous.

11. The method according to claim 10, wherein the at least one
20 foreign T-cell epitope is selected from a natural T-cell epitope and an artificial MHC-II binding peptide sequence.

12. The method according to claim 11, wherein the natural T-cell epitope is selected from a Tetanus toxoid epitope such as P2 or P30, a diphtheria toxoid epitope, a influenza virus hemaglut-
25 tinin epitope, and a *P. falciparum* CS epitope.

13. The method according to any of claims 3-12, wherein the first moiety is a substantially specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific surface antigen.

5   14. The method according to claim 13, wherein the first moiety is a carbohydrate for which there is a receptor on the B-lymphocyte or the APC, such as mannan or mannose or wherein the second moiety is a hapten.

15. The method according to any of claims 3-14, wherein the
10 second moiety is selected from a cytokine, a hormone, and a heat-shock protein.

16. The method according to claim 6, wherein the cytokine is selected from interferon γ (IFN-γ), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-
15 6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF), or an effective part thereof.

17. The method according to claim 15, wherein the heat-shock protein is selected from the group consisting of HSP70, HSP90,
20 and HSC70 or an effective part thereof.

18. The method according to any of claims 3-17, wherein the third moiety is a lipid such as a palmitoyl residue or a myristyl residue.

19. The method according to any of claims 4-7, wherein the OPGL
25 polypeptide has been modified in any one of positions 170-193, any one of positions 198-219, any one of positions 222-247, or in any one of positions 285-317, the amino acid numbering conforming with that of any one of SEQ ID NOs. 2, 4, and 6.

20. The method according to any of the preceding claims, wherein presentation to the immune system is effected by having at least two copies of the OPGL polypeptide, the subsequence thereof or the modified OPGL polypeptide covalently of non-covalently
5 linked to a carrier molecule capable of effecting presentation of multiple copies of antigenic determinants.

21. The method according to any the preceding claims, wherein the OPGL polypeptide, the subsequence thereof, or the modified OPGL polypeptide has been formulated with an adjuvant which
10 facilitates breaking of autotolerance to autoantigens.

22. The method according to claim 21, wherein the adjuvant is selected from the group consisting of an immuno targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine and a mycobacterial derivative; an oil formulation; a polymer; a
15 micelle forming adjuvant; a saponin; an immunostimulating complex matrix; a particle; DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and an encapsulating adjuvant.

23. The method according to any one of claims 1-20, wherein presentation of modified OPGL to the immune system is effected
20 by introducing nucleic acid(s) encoding the modified OPGL into the animal's cells and thereby obtaining *in vivo* expression by the cells of the nucleic acid(s) introduced.

24. The method according to claim 23, wherein the DNA introduced is selected from naked DNA, DNA formulated with charged or
25 uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating a-gents, DNA coupled to an inert carrier molecule, and DNA formu-
30 lated with an adjuvant.

25. A method for treating and/or preventing and/or ameliorating osteoporosis or other conditions characterized by excess bone resorption, the method comprising down-regulating OPGL activity according to the method of any of claims 1-24 to such an extent

5 that the rate of bone resorption is significantly decreased.

26. The method according to claim 25, wherein the rate of bone-resorption is decreased with at least 3%, such as at least 5%, at least 7%, at least 9%, at least 11%, at least 13%, at least 15%, and at least 17%, at least 20%, and at least 30%.

10 27. An OPGL analogue which is derived from an animal OPGL wherein is introduced a modification which has as a result that immunization of the animal with the analogue induces production of antibodies against the OPGL polypeptide.

28. An OPGL analogue according to claim 27, wherein the modifi-
15 cation is as defined in any of claims 1-19.

29. An immunogenic composition comprising an immunogenically effective amount of an OPGL polypeptide which is a self-protein in an animal, said OPGL polypeptide being formulated together with an immunologically acceptable adjuvant so as to break the
20 animal's autotolerance towards the OPGL polypeptide, the composition further comprising a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient.

30. An immunogenic composition comprising an immunologically
25 effective amount of an OPGL analogue according to claim 27 or 28, the composition further comprising a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and optionally an adjuvant.

31. An immunogenic composition according to any of claims 21-23, wherein the adjuvant is selected from the group consisting of the adjuvants of claim 22.

32. A nucleic acid fragment which encodes an OPGL analogue
5 according to claim 27 or 28.

33. A vector carrying the nucleic acid fragment according to claim 32.

34. The vector according to claim 33 which is capable of autonomous replication.

10 35. The vector according to claim 33 or 34 which is selected from the group consisting of a plasmid, a phage, a cosmid, a mini-chromosome, and a virus.

36. The vector according to any of claims 33-35, comprising, in the 5'-3' direction and in operable linkage, a promoter for
15 driving expression of the nucleic acid fragment according to claim 32, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment according to claim 32, and a nucleic acid sequence encoding a terminator.

20 37. The vector according to any of claims 33-36 which, when introduced into a host cell, is integrated in the host cell genome.

38. The vector according to any of claims 33-36 which, when introduced into a host cell, is not capable of being integrated
25 in the host cell genome.

39. The vector according to any of claims 36-38, wherein the promoter drives expression in a eukaryotic cell.

40. The vector according to any of claims 36-39, wherein the promoter drives expression in a prokaryotic cell.

41. A transformed cell carrying the vector of any of claims 33-40.

5 42. The transformed cell according to claim 41 which is capable of replicating the nucleic acid fragment according to claim 32.

43. The transformed cell according to claim 42, which is a microorganism selected from a bacterium, a yeast, a protozoan, or a cell derived from a multicellular organism selected from a

10 fungus, an insect cell, a plant cell, and an mammalian cell.

44. The transformed cell according to claim 43 which is a bacterium of the genus *Escherichia*, *Bacillus*, *Salmonella*, or *Mycobacterium*.

45. The transformed cell according to claim 44, which is an *E.*

15 *coli* cell.

46. The transformed cell according to claim 45, which is a non-pathogenic *Mycobacterium* cell such as *M. bovis* BCG.

47. The transformed cell according to any of claims 41-46, which expresses the nucleic acid fragment according to claim 32.

20 48. The transformed cell according to claim 47, which secretes or carries on its surface, the OPGL analogue according to claim 27 or 28.

49. A composition for inducing production of antibodies against OPGL, the composition comprising

25   a nucleic acid fragment according to claim 32 or a vector according to any of claims 33-38, and

−   a pharmaceutically and immunologically acceptable diluent
    and/or vehicle and/or carrier and/or excipient and/or
    adjuvant.

50. The composition according to claim 49, wherein the nucleic
5 acid fragment is formulated according to claim 25.

51. A stable cell line which carries the vector according to any
of claims 33-40 and which expresses the nucleic acid fragment
according to claim 32, and which optionally secretes or carries
the OPGL analogue according to claim 27 or 28 on its surface.

10 52. A method for the preparation of the cell according to any of
claims 41-48, the method comprising transforming a host cell
with the nucleic acid fragment according to claim 32 or with the
vector according to any of claims 33-40.

53. A method for the identification of a modified OPGL polypep-
15 tide which is capable of inducing antibodies against unmodified
OPGL in an animal species where the unmodified OPGL polypeptide
is a self-protein, the method comprising

−   preparing, by means of peptide synthesis or by molecular
    biological means, a library of mutually distinct modified
20 OPGL polypeptides wherein amino acids have been added to,
    inserted in, deleted from, or substituted into the amino
    acid sequence of an OPGL polypeptide of the animal species
    thereby giving rise to amino acid sequences in the library
    which comprise T-cell epitopes which are and foreign to the
25 animal species,
−   testing members of the library for their ability to induce
    production of antibodies by the animal species against the
    unmodified OPGL, and

- isolating the member(s) of the library which significantly induces antibody production against unmodified OPGL in the animal species.

54. A method for the preparation of an immunogenic composition
5 comprising at least one modified OPGL polypeptide which is capable of inducing antibodies against unmodified OPGL in an animal species where the unmodified OPGL polypeptide is a self-protein, the method comprising

- preparing, by means of peptide synthesis or by molecular
10 biological means, a library of mutually distinct modified OPGL polypeptides wherein amino acids have been added to, inserted in, deleted from, or substituted into the amino acid sequence of an OPGL polypeptide of the animal species thereby giving rise to amino acid sequences in the library
15 comprising foreign T-cell epitopes,
- testing members of the library for their ability to induce production of antibodies by the animal species against the unmodified OPGL, and
- and admixing the member(s) of the library which signifi-
20 cantly induces production of antibodies in the animal species which are reactive with OPGL with a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or diluent and/or excipient, optionally in combination with at least one pharmaceutically and immunologically
25 acceptable adjuvant.

55. The method according to claim 53 or 54, wherein preparation of the members of the library comprises preparation of mutually distinct nucleic acid sequences, each sequence being a nucleic acid sequence according to claim 32, insertion of the nucleic
30 acid sequences into appropriate expression vectors, transformation of suitable host cells with the vectors, and expression of

the nucleic acid sequences, optionally followed by isolation of the expression products.

56. The method according to claim 55, wherein the preparation of the nucleic acid sequences and/or the vectors is achieved by the
5 aid of a molecular amplification technique such as PCR or by means of nucleic acid synthesis.

57. OPGL, a subsequence thereof, or an analogue thereof for use as a pharmaceutical.

58. Use of OPGL or a subsequence thereof for the preparation of
10 an immunogenic composition comprising an adjuvant for down-regulating OPGL activity in an animal.

59. Use of OPGL or a subsequence thereof for the preparation of an immunogenic composition comprising an adjuvant for the treatment, prophylaxis or amelioration of osteoporosis.

15 60. Use of an OPGL analogue for the preparation of an immunogenic composition optionally comprising an adjuvant for down-regulating OPGL activity in an animal.

61. Use of an OPGL analogue for the preparation of an immunogenic composition optionally comprising an adjuvant for the treat-
20 ment, prophylaxis or amelioration of osteoporosis.

62. A method for the treatment, prophylaxis or amelioration of diseases characterized by excess bone resorption in an animal, including a human being, the method comprising administering, to the animal, an effective amount of at least one substance dif-
25 ferent from osteoprotegerin which blocks the stimulatory effect of OPGL on osteoclast activity.

63. The method according to claim 62, wherein the substance is an OPGL-specific antibody or a specifically binding variant thereof.

64. The method according to claim 63, wherein the antibody is
5 polyclonal.

65. The method according to claim 63, wherein the antibody is monoclonal.

66. The method according to any of claims 62-65, wherein the antibody is selected from the group consisting of IgG and IgM.

10 67. The method according to any of claims 62-66, wherein the specifically binding variant of the antibody is selected from the group consisting of
- a Fab fragment,
- a F(ab')$_2$ fragment,
15 - a humanized monoclonal antibody or fragment thereof,
- a di- or multimeric antibody fragment,
- minibodies,
- diabodies, and
- scFv fragments.

20 68. The method according to any of claims 1-22, wherein the OPGL polypeptide, the subsequence thereof or the analogue thereof is administered to the animal via a route selected from the group consisting of the oral, the intramuscular, the the intradermal, the subcutaneous, the subdermal, the intraperitoneal, the oral,
25 the anal, the intravaginal, the sublingual, the buccal, and the intracranial route

69. The method according to claim 68, wherein the effective amount is between 0.5 µg and 2,000 µg of the OPGL polypeptide, the subsequence thereof or the analogue thereof.

70. The method according to any of claims 1-19, wherein presentation to the immune system is effected by administering a nonpathogenic microorganism which has been transformed with the nucleic acid fragment according to claim 32 or the vector according to any of claims 33-40.